Peptidases are affected differently in neocortical regions of brains from patients with Alzheimer's Disease

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Key Words
Alzheimer's disease
Prolylendopeptidase
Aminopeptidase
Frontal cortex

Abstract
Although it is recognized that changes in protease activities may be involved in the etiology of pathological changes in the human brain, there have been few studies on normal and pathological protein catabolism. In the present study the activities of major cortical aminopeptidase and prolylendopeptidase were determined in frontal and medial temporal cortical samples of brains from patients with Alzheimer's disease (AD) and control patients matched for age. Prolylendopeptidase activity was reduced by 45–50% in the frontal and medial temporal cortex of AD patients as compared with controls, whereas major aminopeptidase activity was unaltered. The possibility that the changes in peptidase activities in AD are age-dependent was studied in postmortem neocortical samples from 18 AD patients who had died between 55 and 90 years of age. Prolylendopeptidase activity was lower in patients who died at a relatively early age than in patients who died at a relatively old age. This underscores the notion that, with respect to age, there may be heterogeneity in neurochemical changes in AD.

Introduction

Alzheimer's disease (AD) is the most prevalent neurodegenerative disorder causing dementia and affects 5% of the population over 65 years of age [1]. This disease is characterized by large numbers of neuritic plaques and neurofibrillary tangles, and is associated with many biochemical alterations, such as reduced activities of choline acetyltransferase and dopamine-β-hydroxylase [2–5], altered phosphorylation of cytoskeletal proteins [6], and abnormal protein processing leading to deposition of β-amyloid peptide [7]. How these biochemical alterations relate to each other and to the cause and pathogenesis of AD is not yet understood. The observation that the formation of neuritic plaques results from abnormal protein deposition prompted investigators to look for abnormalities in protein catabolism in AD. For instance, serine proteases [8] (various complement factors), serine protease inhibitors [9], and calpain [10] have been identified as components of neuritic plaques. Ubiquitin, a small protein involved in energy-dependent proteolysis, is present in neurofibrillary tangles [11]. Several studies on peptidase activities in AD have been carried out. Mantle et al. [12] did not find changes in major aminopeptidase activity in neocortical samples from AD patients. Likewise, Kawashima et al. [13] and Nilsson et al. [14] failed to find

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Table 1. Patient information

Cases	Age, sex	Brain weight, g	Postmortem delay, min	Cause of death
Controls				
1	60 M	1,350	360	septic shock following aorta valve implantation
2	65 M	1,310	315	heart failure
3	66 F	1,100	375	postoperative heart failure
4	71 F	1,240	305	sepsis and cardiogenic shock
5	73 M	1,340	255	cerebral infarct
AD				
1	55 M	1,020	180	pneumonia, cachexia and dehydration
2	64 M	1,210	330	collum fracture (died after operation)
3	65 M	1,360	240	pulmonary embolism, dehydration
4	68 F	895	345	bronchopneumonia
5	70 M	1,075	270	cachexia
6	72 M	1,040	285	pneumonia and cachexia
7	79 F	1,010	245	bronchopneumonia and cachexia
8	81 F	910	225	pneumonia and heart disease
9	84 F	920	205	pneumonia
10	88 F	1,070	225	pneumonia
11	81 F	1,130	330	suffocation
12	85 F	1,020	135	intestinal bleeding
13	87 F	950	235	apnea following postoperative irreversible hypotension
14	89 F	940	250	pneumonia
15	88 F	960	225	lung infection
16	89 F	1,050	200	unknown
17	90 F	840	230	cachexia and dehydration
18	90 F	1,000	270	acute myocard infarct, massive pulmonary embolism

changes in calpain activity in AD brains. Aoyagi et al. [15] measured aminopeptidase, dipeptidylpeptidase, cathepsin B and serine protease activities in occipital cortex from AD patients and observed decreased kallikrein activity and increased prolylendopeptidase activity. The fact that only these two proteases were affected in AD may be related to the age distribution of the patients examined, since it has been observed that neurochemical markers of AD are influenced by age [16]. It is thus important to consider age when studying neurochemical changes in AD. Major aminopeptidase is the most active aminopeptidase and prolylendopeptidase is the most active serine protease of brain tissue [17, 18]. Their ubiquitous nature suggests that they serve a fundamental role in protein turnover. So far these enzymes were studied in relatively old AD patients only [12, 15]. Therefore it was considered important to determine these enzyme activities in AD patients with respect to age. Firstly, major aminopeptidase and prolylendopeptidase activities were determined in neocortical autopsy samples from relatively young AD and control patients matched for age and postmortem delay. Secondly, these enzyme activities were determined in tissue from AD patients who died between 55 and 90 years of age.

Materials and Methods

Brain tissues were obtained from the Netherlands Brain Bank. Eighteen patients (5 male, 13 female; age range 55–90 years) were selected that had been clinically diagnosed as probable Alzheimer cases according to the NINCDS–ADRDA criteria [19] (for patient information see table 1). They had a score of 6–7 on the Global Deterioration Scale of severity of dementia [20]. The clinical diagnosis of AD was verified by postmortem neuropathological examination of formalin-fixed specimens. Five AD patients were selected for the first experiment [4 male, 1 female; mean age (SD) 68 (3); postmortem delay (SD) 294 (43) min; No. 2–6, table 1]. Five patients with no evidence of dementia and no history of other neurological or psychiatric disorders were selected as normal controls [3 male, 2 female; mean age (SD) 67 (5); postmortem delay (SD) 322 (48) min; No. 1–5, table 1]. Tissue blocks of about 1 g fresh weight were excised from the frontal superior gyrus and the medial temporal gyrus. The leptomeninges were removed and the samples were rapidly frozen in liquid nitrogen. The frozen samples were stored at −80 °C until use. Brain

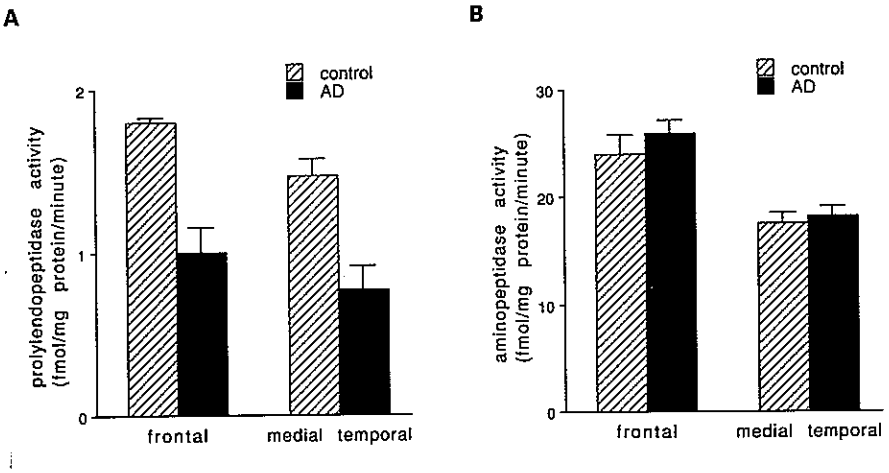

Fig. 1. Prolylendopeptidase (**A**) and major aminopeptidase (**B**) activities in medial temporal and frontal cortex of AD and control patients matched for age. Each group consisted of 5 patients.

tissue samples were homogenized in 9 volumes 0.32 *M* sucrose, 1 m*M* EGTA, 50 m*M* Tris, pH 7.4, in a Potter-Elvehjem Teflon-glass homogenizer (12 up-and-down strokes, 700 rpm, 0.25 mm clearance). The homogenates were centrifuged at 100,000 *g* for 60 min and the resulting membrane-free fraction was frozen in liquid nitrogen and stored at −80 °C until determination of peptidase activities.

Assay for the Determination of Major Aminopeptidase Activity

Aminopeptidase activity was measured with a modification of a previously reported assay [21]. Vasopressin iodinated in the Tyr^2 position was used as peptide substrate (Amersham, UK). Two incubations were carried out, either in the presence of 32 μ*M* puromycine or in the absence of the inhibitor. The difference between the release of Tyr in these two incubations was used as an index of puromycin sensitive (major) aminopeptidase activity. Iodinated VP was incubated with 2.5 μg cytosolic protein for 2.5 min in 100 μl of a buffer consisting of 50 m*M* Tris-HCl (pH 7.4), 1 mg human serum albumin/ml and 1 m*M* dithiotreitol. Conditions were chosen such that less than 20% of the substrate was converted. The reaction was stopped by the addition of 0.9 ml 0.1 *M* NaOH. Iodinated Tyr was separated from the substrate by the addition of 50 mg Amberlite XAD-2 (BDH, Pool, UK) prewetted with 50 μl ethanol. The tubes were kept in a rotary tumbler (Luckham, Burgess Hill, UK) for 30 min to allow VP to bind to the beads. Beads were spun down at low speed and the Tyr containing supernatants were pipetted off.

Assay for the Determination of Prolylendopeptidase Activity

The prolylendopeptidase assay was based on the method of Davison et al. [22]. Iodinated VP was used as a substrate. Davison et al. determined N-terminal conversion of the peptide. In the present study, C-terminal conversion of the peptide was assessed as the reduced binding of radioactivity to excess C-terminus recognizing antibody. The antibody has been characterized previously [23]. Iodinated VP (10 p*M*) was incubated with 12.5 μg of cytosolic protein for 5 min in 100 μl of a buffer consisting of 50 m*M* Tris-HCl (pH 7.4), 1 mg HSA/ml and 1 m*M* 1,10-phenanthroline. 1,10-Phenanthroline was present during the incubation to inhibit aminopeptidase activity. Conditions were chosen such that less than 20% of the substrate was converted. The reaction was stopped by addition of 0.4 ml 1 *M* acetic acid and boiling for 5 min. Flocculent material was removed by centrifugation (50×10^3 *g*, 30 min). The supernatants were evaporated under vacuum (Speed Vac Concentrator). The freeze-dried residues were dissolved in 200 μl of a Veronal/HSA buffer. Diluted anti-VP antiserum (1:1,000; 25 μl) was added to 50-μl aliquots. After 72 h of incubation bound and free radioactivity were separated by the addition of 100 μl of a suspension of dextran-Ficoll-coated charcoal [24]. Charcoal was spun down and the supernatant was removed by suction. Charcoal pellets were counted in a well-type γ-counter.

Results

Prolylendopeptidase and major aminopeptidase activities were determined in autopsy samples of the frontal superior gyrus of 5 AD patients and control subjects matched for age and postmortem delay. Total aminopeptidase activity consisted of puromycin-sensitive aminopeptidase activity only, that is puromycin-insensitive activity was not detected. Prolylendopeptidase activity was reduced by 44 and 48% in the frontal and medial temporal cortex of the 5 AD patients as compared with the 5 control patients matched for age (p values < 0.01, fig. 1a). Aminopeptidase activity was not affected in the 5 AD patients (p values > 0.05, fig. 1b).

Peptidase activities were determined in medial temporal cortex samples of 18 AD patients who had died between 55 and 90 years of age. Using a split half design, we tested whether there was an age-related difference in AD patients with respect to peptidase activities. The older AD

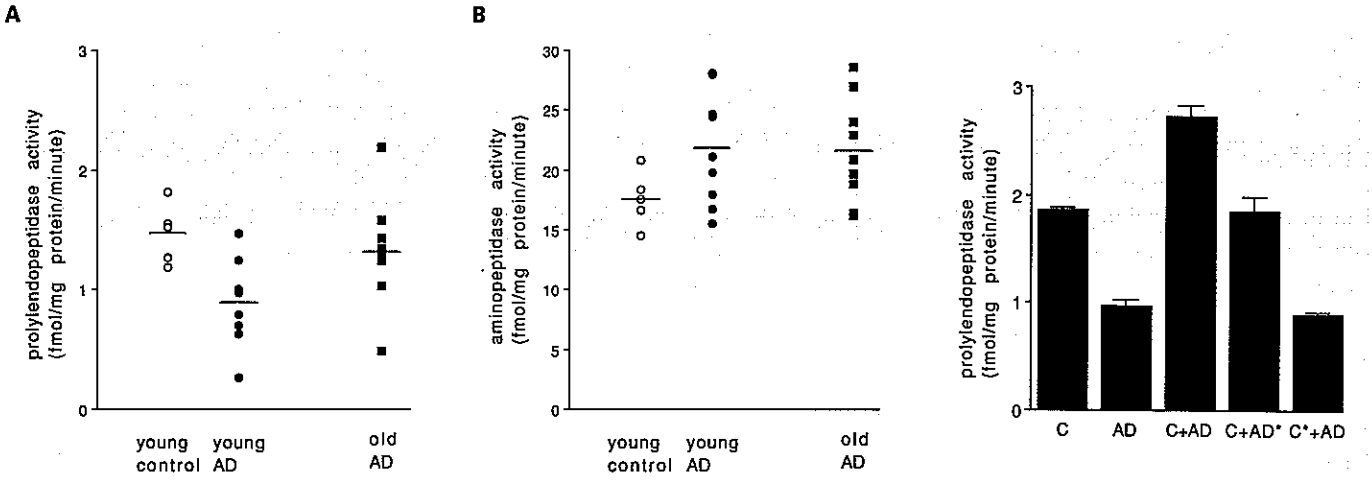

Fig. 2. Prolylendopeptidase (**A**) and aminopeptidase (**B**) activities in medial temporal cortex of control patients (o), AD patients younger than 84 years (•) and AD patients older than 84 years (■).

Fig. 3. Prolylendopeptidase activity in cytosolic fractions of frontal cortex of control patients and AD patients, combined fractions (C+AD), combined control and heat-denatured AD fractions (C+AD*) and combined heat-denatured control and Alzheimer's disease fractions (C*+AD). Heat denaturation was achieved by heating at 65 °C for 30 min.

patients (> 84 years) had a significantly higher prolylendopeptidase activity than the younger ones (< 84 years) ($p < 0.05$, fig. 2a). The older AD patients did not differ from the 5 control patients ($p > 0.05$), but the younger AD patients did ($p < 0.01$, fig. 2a). Comparisons with older control patients were not possible because material from older control patients was not available. Aminopeptidase activity was unrelated to age in AD patients ($p > 0.05$, fig. 2b).

To investigate whether the lower prolylendopeptidase activity was caused by either the presence of an endogenous inhibitor or the absence of a cofactor in cytosolic fractions of neocortical tissue from AD patients, cytosolic fractions prepared from AD material were combined with cytosolic fractions prepared from control material. Combination of cytosolic fractions did not result in a decrease in the sum of activities ($p > 0.05$, fig. 3). Heat-denatured cytosolic fractions of AD patients did not affect the prolylendopeptidase activity of cytosolic fractions of controls or vice versa (p values > 0.05, fig. 3).

Discussion

In the present study two peptidase activities were measured in postmortem brain samples of patients with AD and of control patients. Prolylendopeptidase was determined radioimmmunochemically and aminopeptidase radiometrically with vasopressin iodinated in the Tyr^2 position. We used these methods on the basis of our experience with the measurement of the conversion of vasopressin by membrane and cytosolic fractions and with the radioimmunoassay of vasopressin [21, 24, 25]. Since vasopressin is not present in brain cortex, this study is not of relevance to the in vivo conversion of vasopressin. It has been shown previoulsy that vasopressin is converted by purified major cytosolic aminopeptidase [17] and prolylendopeptidase [18]. In addition, vasopressin is converted by both N- and C-terminal degrading enzymes when incubated with a cytosolic fraction [21]. When assessing prolylendopeptidase activity, aminopeptidase activity was inhibited with 1 m*M* 1,10-phenanthroline. The possibility that C-terminal conversion of vasopressin may have affected the determination of N-terminal conversion can be excluded, because conditions were chosen such that only a few percent of vasopressin was converted C-terminally, and N-terminal fragments of vasopressin can still serve as a substrate for the aminopeptidase.

Prolylendopeptidase activity was reduced by 45–50% in the frontal and medial temporal cortices of AD patients as compared with tissue from controls matched for age. In contrast, major aminopeptidase activity was not decreased in the frontal and medial temporal cortices of AD patients as compared with control patients. Since major aminopeptidase and prolylendopeptidase are both cytosolic peptidases [17, 18], the decrease in the activity

of the former enzyme is probably not related to neuronal death.

Prolylendopeptidase also appeared to be affected by the age of the AD patients. This can be explained in two possible ways. Firstly, older patients may have died earlier in the disease. This possibility is not supported by the observation that the diagnosed duration of disease was not significantly different between the younger and older AD patients. Nor was there a correlation between the duration of disease and prolylendopeptidase activity. Secondly, the age dependence of prolylendopeptidase activity may reflect a nosological distinction between late and early onset AD. Rossor et al. [16] have recently reported on the age dependence of neurochemical markers of AD. The present findings corroborate these observations for prolylendopeptidase activity. No effect of age was found on major aminopeptidase activity. The suggestion that an effect on major aminopeptidase activity may have been missed previously [12] can now be excluded.

Prolylendopeptidase activity in the brains of AD patients may be reduced by three possible mechanisms: (1) reduced synthesis of the enzyme; (2) damage to the enzyme; (3) inhibition of the enzyme by an endogenous component or the absence of a cofactor. The latter possibility was excluded by the observation that combination of cytosolic fractions of AD and control patients did not result in decreases or increases in the sum of activities which would have occurred if an inhibitor or the absence of a cofactor were the cause of the reduced enzyme activity. Nor did heat-denatured cytosolic fractions from AD patients contain an inhibitor of prolylendopeptidase activity.

In striking contrast to our findings Aoyagi et al. [15] found an increase in prolylendopeptidase activity in occipital cortex from AD patients. This cannot be readily explained. It may be related to the particular sample of patients selected by Aoyagi et al. [15] and heterogeneity within AD cases; these investigators did not diagnose their patients according to the NINCDS-ARDRA criteria [19] and used relatively old patients. In the present study no change was observed in prolylendopeptidase in brain tissue from relatively old AD patients.

AD is characterized by a disturbance in protein catabolism (deposition of proteins and abnormal protein cleavage) and changes in protein degrading enzymes are probably involved. Whether the disturbance found in the present study is a causative factor in AD and how peptidases participate in the etiology of AD needs further investigation. Prolylendopeptidase is a serine protease and the reduction in prolylendopeptidase activity observed may be representative of other serine proteases.

Some of the enzymes affected in AD are associated with well-defined transmitter systems (e.g., choline acetyltransferase [5]), whereas other enzymes, such as the presently studied peptidases, are of ubiquitous nature. It is likely that these reduced enzyme activities have a common cause. Some enzyme activities may be reduced by dephosphorylation, since tyrosine kinase activity is reduced in AD [26]. This is not a likely explanation for the changes in the peptidase activities, since the activity of these enzymes is not regulated in such a way. It would be interesting to try to find a common denominator for the changes in enzyme activities observed. Factors that may reduce enzyme activities are changes in cellular metal concentrations [27] and free radical formation [28]. Whether one of these factors is the common denominator is far from certain, but tracing the cause of the changes in enzyme activities may be a fruitful approach to the elucidation of the etiology of AD.

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